Journal of Surgical Oncology 1997;66:173–178

Low Level c-*myc* Gene Amplification in Gastric Cancer Detected by Dual Color Fluorescence In Situ Hybridization Analysis

SEIJI SUZUKI, MD,* TOSHIHIRO TENJIN, MD, HIDEHIRO WATANABE, MD, SHINJI MATSUSHIMA, MD, TETSUO SHIBUYA, MD, AND SHIGEO TANAKA, MD
Second Department of Surgery, Nippon Medical School, Tokyo, Japan

Background and Objectives: By using the dual color fluorescence in situ hybridization analysis, the amplification of c-*myc* gene can be detected in the tumor tissue samples obtained from patients with gastric cancer, and the relationship between the molecular cytogenetic change and the clinical stage or histological type may be clarified.
Method: The tumor tissue samples were obtained from 21 patients with gastric cancer. Simultaneous detection of signals from the chromosome 8 centromere and c-*myc* gene in each cell after hybridization with appropriate probes was carried out on 50–200 tumor cells in each case.
Result: Chromosome 8 polysomy was found in 10 patients. The average centromere 8 copy number was significantly higher in differentiated (2.7) than in undifferentiated (2.3) types of gastric cancer. However, there was no significant difference in occurrence of polysomy 8 between early and advanced cancer. The relative gain (1.1–1.9) of c-*myc* copy number was found in all 21 cases. There was no significant difference in the fraction of cells with c-*myc* gene amplification between early (pT1) and advanced (pT2–4) carcinomas.
Conclusion: We conclude that the present dual color fluorescence in situ hybridization of gastric cancer may be useful in the evaluation of low level c-*myc* gene amplification, which is difficult to detect by Southern blotting, and may be applicable to the diagnosis of early gastric cancer.
J. Surg. Oncol. 1997;66:173–178.

KEY WORDS: gastric cancer; dual color FISH; chromosome 8; c-*myc* gene; gene amplification

INTRODUCTION

Various genetic alterations and chromosomal aberrations are involved in carcinogenesis and cancer progression [1–5]. Most of the genetic information of solid tumors is derived from analyses of total DNA and RNA extracted from the mixtures of tumoral and adjacent normal tissue. Such analyses cannot clarify the genetic changes and chromosomal aberrations occurring in each tumor cell.

However, by using the dual color fluorescence in situ hybridization (FISH) analysis, the deletion or amplification of the target gene or the specific locus of a certain chromosome, in addition to the numerical chromosomal aberrations, can be detected even in the interphase of cell division [6–9]. Furthermore, the dual color FISH analysis can be applied for detection of low level gene amplification, which is difficult to detect by the currently available molecular biological techniques such as Southern blotting [8].

The c-*myc* gene, localized in the chromosomal region 8q24 and containing about 5,000 base pair, may play an important role in cell proliferation and differentiation and may induce apoptosis of cells under certain conditions [10].

*Correspondence to: Seiji Suzuki, MD, The Second Department of Surgery, Nippon Medical School, Sendagi 1-1-5, Bunkyo-Ku, Tokyo 113, Japan. Fax: (011)-81-3-5685-0985.
Accepted 27 July 1997

Although the incidence of cells with c-*myc* DNA amplification is low (less than 12%) in carcinogenesis of the stomach [11–14], Onoda et al. [15] reported that c-*myc* messenger RNA (mRNA) and c-*myc* product (protein) were overexpressed in the early stage of stomach cancer: c-*myc* mRNA was overexpressed in 54.8% of the advanced and in 90.0% of the early cases. However, they speculated that the overexpression of c-*myc* mRNA was not caused by c-*myc* DNA abnormality or amplification.

By dual color FISH analysis, Sauter et al. [9] defined chromosome 8 polysomy as an average centromere 8 copy number above 2.3, and they found that the relative gain of c-*myc* gene copy number can be determined by comparing the number of c-*myc* signals with the number of chromosome 8 centromeric signals in each tumor cell analyzed. The c-*myc* gene copy number gain (c-*myc* DNA amplification) in their study on bladder cancer was defined as more c-*myc* signals than centromere 8 signals in more than 10% of all scored cells.

In the present study, we examined the low level c-*myc* gene amplification, in addition to numerical aberrations of chromosome 8, in cancer of the stomach to clarify the relationship between the molecular cytogenetic change and the clinical stage or histological type.

TABLE I. Clinicopathological Findings, Chromosome 8 Copy Number, and c-*myc* Gene Amplification in 21 Cases With Gastric Cancer*

Case	[A]	[B]	[C]	[D]	[E]	[F]	[G]
1	T1N0M0	sig	2.0	5.0	3.7	1.9	85.0
2	T1N0M0	sig	2.1	3.2	2.9	1.5	54.8
3	T1N0M0	tub1	2.2	24.1	3.6	1.6	66.7
4	T1N0M0	tub1	2.7	51.0	5.0	1.9	89.0
5	T1N0M0	tub2	2.1	7.0	2.3	1.1	19.0
6	T1N0M0	tub1	3.2	76.3	4.7	1.3	54.4
7	T2N2M0	por2	2.4	31.5	3.0	1.4	37.5
8	T2N1M0	tub1	2.1	12.5	3.2	1.5	12.5
9	T2N2M0	por2	2.3	23.5	3.0	1.3	39.2
10	T2N1M0	tub2	3.0	66.5	5.4	1.6	64.5
11	T2N1M0	tub1	3.1	59.3	5.3	1.7	92.0
12	T2N2M0	por1	2.4	21.7	4.1	1.8	83.3
13	T2N2M0	por2	2.1	11.0	3.0	1.4	61.5
14	T2N1M0	por2	3.1	72.0	4.2	1.4	60.5
15	T3N1M0	por1	2.1	7.0	3.3	1.6	71.0
16	T3N1M0	tub1	3.3	76.0	4.8	1.4	76.0
17	T3N1M0	tub1	2.4	29.2	4.2	1.7	83.0
18	T3N2M0	sig	2.1	14.0	3.4	1.7	81.0
19	T4N1M1	sig	2.3	22.0	3.8	1.7	84.0
20	T4N2M1	por2	2.1	11.0	3.0	1.5	48.0
21	T4N2M0	por2	2.7	32.0	4.3	1.7	79.0

*[A], pTNM; [B], histological classification: sig: signet-ring cell; tub: tubular carcinoma; por: poorly differentiated carcinoma; [C], average centromere 8 copy number; [D] percent of chromosome 8 polysomy; [E], average c-*myc* gene copy number; [F], average ratio of c-*myc* gene copy number to centromere 8 copy number; [G], percent of c-*myc* gene amplified cell.

MATERIALS AND METHODS

Tumor tissue samples were obtained from 21 gastric cancer patients who underwent gastrectomy. The patients consisted of 18 men and 3 women, between 42 and 82 years old (mean age, 69.3 years). The stage of each cancer was determined according to the TNM classification of the International Union against Cancer (Table I, column [A]). The histological diagnosis was determined according to the Japanese Classification of Gastric Carcinoma [16]: signet-ring cell carcinoma (sig) in 4 cases, tubular adenocarcinomas (tub1 and tub2) in 10 cases, and poorly differentiated adenocarcinomas (por1 and por2) in 7 cases (Table I, column [B]). There were 10 cases of differentiated (tub1 and tub2) and 11 cases of undifferentiated carcinomas (por1, por2, and sig) in the present series.

Sample Preparation

Nuclear suspensions from surgical specimens were obtained by mechanical disaggregation according to the procedures described below. Each specimen was minced and put into phosphate buffered saline (PBS). The solution was filtered through a gauze mesh, and centrifuged at 1,000 rpm for 5 min. The resulting sediment was suspended in PBS, and the suspension was filtered through a 100-μm nylon mesh. The filtrate was centrifuged again, after which the sediment was suspended in 0.75 M KCl solution, and the suspension was centrifuged. The sediment was then suspended in Carnoy solution (methanol/acetic acid, 3:1) for 5 min, and the suspension was centrifuged. The sediment was finally suspended in a small amount of Carnoy solution, and a drop of the suspension was placed onto a glass slide. After air drying, each prepared slide was stored at −20°C until hybridization.

Fluorescence In Situ Hybridization

FISH analysis was carried out as described by Pinkel et al. [17], with some modifications. Before hybridization, each slide was immersed in a 2 × SSC solution (1 × SSC: 0.15 M NaCl, 15 mM sodium citrate) for 30 min and then in a 70% acetic acid solution for 2 min. After drying, each slide was washed in fresh PBS, and dehydrated in a series of ethanol solutions (70, 85, and 100%). After drying, each slide was immersed in 0.25% acetic acid/0.1 M Tris-HCl solution for 10 min and washed in fresh 2 × SSC solution.

Each slide preparation was then denatured in 70% formamide plus 2 × SSC (pH 7.4) at 74°C for 4 min. Immediately after this denaturing, the slide preparation was cooled in a 70% ethanol solution at −20°C for 5 min, and dehydrated in the ethanol series (70, 85, and 100%) at 4°C for 2 min each. The slide preparation was air-dried, and 9.5 μl from a probe mixture containing of 7 μl

of hybridization buffer (dextran sulfate, 70% formamide, 2 × SSC, pH 7.0), 1 μl of 8 alpha satellite repeat DNA probe (labeled with Spectrum Green to 8 alpha satellite centromere [Vysis, Inc., Chicago, IL]), 1 μl of 8q24 locus specific (c-*myc* gene) cosmid probe (labeled with Spectrum Orange to 8q24.2–q24.3 region [Vysis, Inc.]), and 1 μl of distilled water was applied to the target area of the preparation, after the probe mixture had been denatured at 75°C for 10 min and then immediately cooled in ice water for 5 min.

The target area was then covered with an 18 × 18 mm glass coverslip. After overnight hybridization at 37°C in a wet chamber, each slide was washed twice in a 2 × SSC solution at 42°C. Next, each slide was immersed in a PNM buffer (15% non-fat dry milk in 0.1 M NaH_2PO_4, 0.1 Na_2HPO_4 and 0.1% NP-40, pH 8.0) at 42°C for 10 min. The nuclei were counterstained with 125 ng/ml of DAPI (diamidino-phenylindole, Vysis, Inc.) solution. Normal lymphocytes and normal gastric epithelial cells were used as the control.

Detection of Signals

Using an Olympus fluorescence microscope with a single band-pass filter, each slide was scored for the number of hybridized signals from the chromosome 8 centromere (centromere 8) and the 8q24 locus for 50–200 nuclei. In addition, the average centromere 8 copy number per cell, the average c-*myc* copy number per cell, the average ratio of c-*myc* copy number to centromere 8 copy number per cell, and the fraction (percentage) of c-*myc* gene amplified cells were determined.

According to Sauter et al. [9], the c-*myc* gene amplification was defined as more c-*myc* signals than centromere 8 signals in more than 10% of scored cells, and chromosome 8 polysomy was defined as an average centromere 8 copy number above 2.3.

RESULTS

Control Study

Normal lymphocytes and normal epithelial cells of the stomach were examined by the dual color FISH analysis for the chromosome 8 centromere copy number and c-*myc* gene copy number. The average centromere 8 copy number per cell was 1.96 in the normal lymphocytes and 2.02 in the normal epithelial cells of the stomach, and the average c-*myc* gene copy number per cell was 2.08 and 1.97, respectively. The average ratio of c-*myc* gene copy number to the centromere 8 copy number per cell was 1.03 in the normal lymphocytes and 1.04 in the normal epithelial cells of the stomach. Thus, most of the control lymphocytes and gastric epithelial cells showed two centromere 8 signals and two c-*myc* gene signals per cell, and the predominant pattern of centromere 8 copy number/c-*myc* gene copy number (per cell) was 2/2, i.e., 87.0% in the normal lymphocytes and 90.0% in the normal gastric epithelial cells.

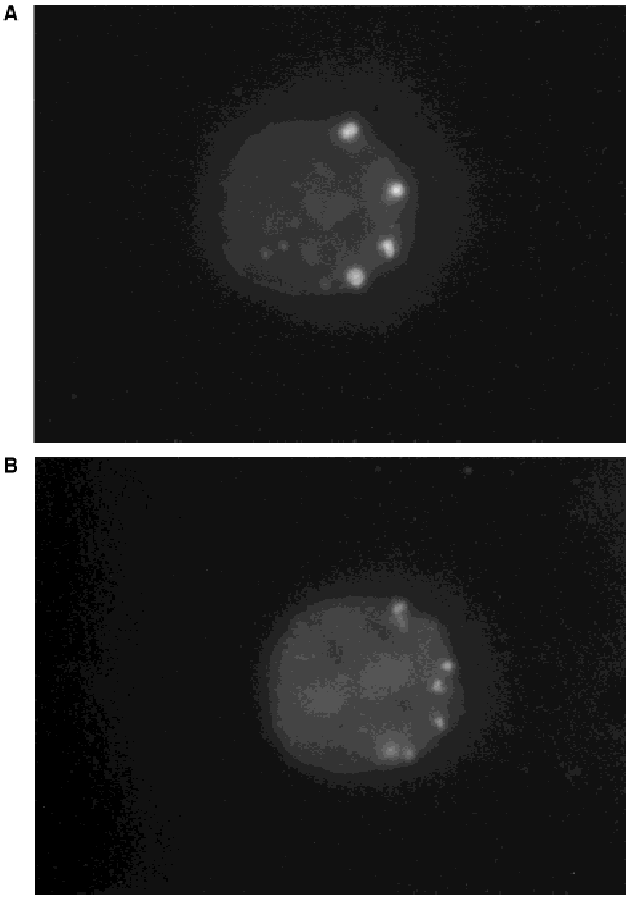

Fig. 1. Photographs of the nucleus of a tumor cell obtained by dual color FISH analysis in a patient with gastric cancer (Case 10) (×400). The pattern of 8 alpha centromere/8q24 signals is 4/6, suggestive of low level c-*myc* gene amplification. **A:** Four green signals from hybridized chromosome 8 alpha centromeric probe in the nucleus. **B:** Six red signals from hybridized 8q24 cosmid probe in the same nucleus.

Chromosome 8 Copy Number

The copy numbers detected by the probe specific to the 8 centromere were four (Fig. 1A) and the copy numbers detected by the probe specific to the 8q24 locus were six (Fig. 1B) in the nucleus of a tumor cell of Case 10.

Chromosome 8 polysomy showing the average centromere copy number per cell above 2.3 was found in 10 of 21 cases (47.6%), as shown in Table I, column [C]. The correlative analysis of the average centromere 8 copy number to the stage (depth of tumor invasion) revealed no significant difference in the average centromere 8 copy number between pT1 and pT2–4. There was also no significant difference in the average centromere 8 copy number between tumors with and without lymph node involvement. However, the average centromere 8 copy number was significantly higher in differentiated (2.7)

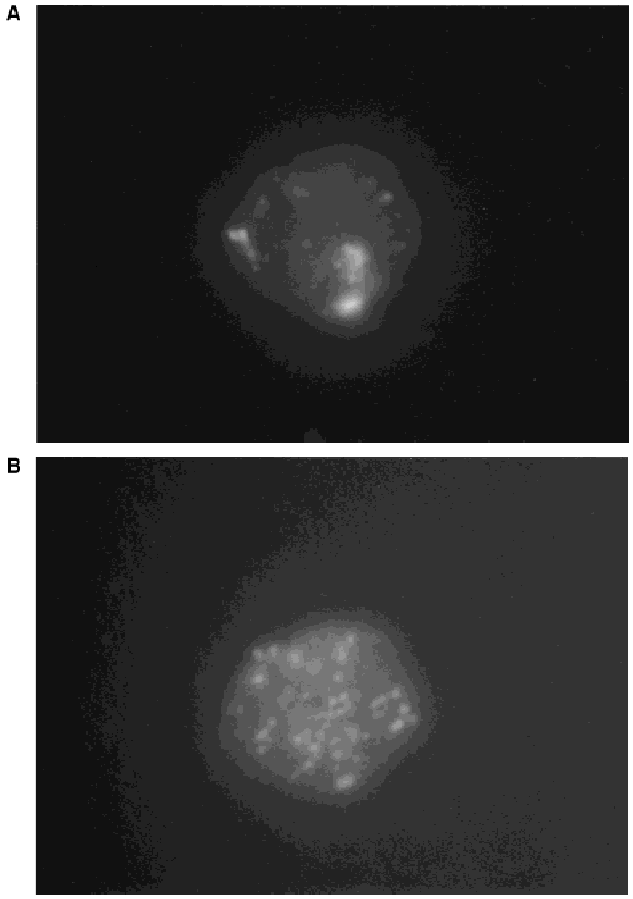

Fig. 2. Photographs of the nucleus of another tumor cell in the same patient as in Figure 1 (Case 10) (×400). The ratio of 8q24 cosmid to 8 alpha centromere signals is suggestive of high level c-*myc* gene amplification. **A:** Three green signals from hybridized chromosome 8 centromere probe in the nucleus. **B:** Red signals (over 30 in number) from hybridized 8q24 cosmid probe, distributed diffusely in the nucleus, suggest double minute pattern.

than undifferentiated (2.3) types of cancer ($P < 0.05$, Student's *t* test).

Low Level c-*myc* Gene Amplification

The relative gain of the c-*myc* gene copy number (c-*myc* gene amplification) in gastric cancer was determined by comparing the number of c-*myc* signals with the number of chromosome 8 centromeric signals in each of scored cells. The average ratio of c-*myc* gene copy number to centromere 8 copy number was between 1.1 and 1.9 (low level gains according to Sauter et al. [9]) in 21 carcinomas in the present series, as shown in Table I, column [F] and Figure 1. In this patient (Case 10), however, another nucleus obtained from the same cancer demonstrated high level c-*myc* gene amplification, i.e., a large number (over 30) of 8q24 cosmid signals distributed diffusely in the nucleus (Fig. 2). It was thought that the diffuse distribution of c-*myc* gene signals, as shown in Figure 2, was compatible with double minute (DM) pattern, reflecting the intratumor heterogeneity.

The percent of cells with c-*myc* gene amplification (c-*myc* signals > centromere 8 signals per cell) was between 12.5 and 89.0% of all scored cells in 21 patients (Table I, column [G]). There was no significant difference in the fraction (%) of cells with c-*myc* gene amplification between pT1 and pT2–4 tumors, and between differentiated and undifferentiated carcinomas. The average percent of cells with c-*myc* gene amplification was 71.9% in the chromosome 8 polysomy group (10 tumors) and 56.7% in the disomy group (11 tumors). The difference of both percentages was significant ($P < 0.05$, Student's *t* test).

DISCUSSION

The frequency of chromosome 8 polysomy showing the average centromere 8 copy number per cell above 2.3 was 47.6% (10 in 21 cases of stomach cancer) in the present series, and was similar to that (47.1%) in bladder cancer reported by Sauter et al. [9].

Rao et al. [18] detected trisomy 8 in 4 of 10 gastric cancer cases, and Abe [19] reported that the increase of the chromosome 17 centromere copy number was significantly greater in the mucosal than advanced stage tumors. Abe also found that the chromosome 7 centromere copy number was significantly higher in the group with than in the group without lymph node involvement. We found no correlation between the chromosome 8 centromere copy number and the depth of tumor invasion of stomach cancer, although a marked polysomoid change was seen in a case (Case 4) of the early cancer (sm).

The present findings suggested that numerical aberrations of chromosome 8 were common events in the carcinogenesis of the stomach but did not play an important role in the progression of cancer. The significant difference in the average centromere 8 copy number between differentiated and undifferentiated types of stomach cancer suggested that the chromosome 8 aberrations play a role in the differentiation of cancer.

Chromosome 8 disomy and trisomy in the copy number category, according to Waldman et al. [20], were dominant in the present series of gastric cancer patients. Although monosomy in the copy number category of the chromosome 17 had been dominant in our previous study [21] on stomach cancer, chromosome 8 monosomy was seldomly seen in the present study. Thus, the dominant type of numerical chromosome aberrations appears to vary among chromosomes of gastric cancer cells; disomy and trisomy were dominant in chromosome 8, whereas monosomy and disomy were dominant in chromosome 17.

Nomura et al. [11] and Mor et al. [13] reported a low frequency (11.1 and 7.2%, respectively) of c-*myc* gene amplification by Southern blotting in stomach cancer, whereas a low level c-*myc* gene amplification (copy number gain ranging from 1.2 to 1.9) could be detected

by dual color FISH analysis in all of our 21 patients with gastric cancer. Although Sauter et al. [9] found that a low level c-*myc* gene amplification was more frequent in advanced stage than in early stage of bladder cancer, we found no correlation between the c-*myc* gene amplification and the depth of tumor invasion of stomach cancer.

Kallioniemi et al. [8] used dual color FISH for analysis of *ERBB2* gene copy number per cell and the level of amplification (defined as the ratio of *ERBB2* copy number to chromosome 17 copy number) in breast cancer. They found that cancer cells with a ratio greater than 2.0 could be detected by Southern blotting as having the amplified *ERBB2* gene. By using dual color FISH analysis on cancer of the stomach, we easily detected a low level c-*myc* DNA amplification that could not be detected by Southern blotting.

According to Onoda et al. [15], c-*myc* mRNA and c-*myc* protein were overexpressed even at the early stage of gastric carcinomas. Because c-*myc* DNA was not always amplified in those carcinomas, they speculated that the overexpression of c-*myc* mRNA and c-*myc* product originated from the abnormality of the genetic signal from mRNA to protein.

The higher percentage of c-*myc* gene amplified cells in polysomy than disomy in our study suggests that low level gene amplification accompanied numerical aberrations of chromosome 8. Furthermore, the low level gene amplification detected by FISH analysis may originate from chromosomal instability. The chromosomal 8 copy number aberrations and the low level c-*myc* gene amplification were observed even at the early stage of gastric cancer. Thus, dual color FISH analysis, by using biopsied specimens, may play an auxiliary role in the diagnosis of early gastric cancer. In addition, it may be applicable to follow-up studies of the remnant stomach after partial gastrectomy, in precancerous lesions of the stomach, and in evaluation of the effect of chemotherapy.

Because the biological significance of low level c-*myc* DNA amplification in cancer cells and apoptosis remains unknown, further extensive investigations are needed for its clarification.

CONCLUSION

Chromosome 8 polysomy showing the average centromere copy number per cell above 2.3 was found in 10 of 21 patients. There was no significant difference in the average centromere 8 copy number between early and advanced carcinomas, but polysomy 8 was significantly higher in differentiated than undifferentiated carcinomas.

The relative gain of c-*myc* gene copy number was found in all 21 cases and ranged from 1.1 to 1.9. The fraction (%) of cells with c-*myc* gene amplification ranged from 12.5 to 89.0% of all scored cells. There was no significant difference in the fraction of cells with c-*myc* gene amplification between early (pT1) and advanced (pT2–4) carcinomas of the stomach. The average fraction of cells with c-*myc* gene amplification was 71.9% in the chromosome 8 polysomy group (10 tumors) and 56.7% in the disomy group (11 tumors); the difference being significant.

Thus we conclude that the present dual color FISH analysis was a useful method for simultaneous identification of chromosomal aberrations and c-*myc* gene amplification of tumor cells even during the interphase of cell division and might offer an important clue to the relationship between molecule cytogenetic change and clinical stages or histological types. In addition, the dual color FISH analysis allows evaluation of low level c-*myc* gene amplification, which is difficult to detect by Southern blotting.

ACKNOWLEDGMENT

We thank Miss Motoko Yamamoto for her skillful technical assistance.

REFERENCES

1. Rodriguez E, Sreekantaiah C, Chaganti RSK: Genetic change in epithelial solid neoplasia. Cancer Res 1994;54:3398–3406.
2. Ochi H, Douglass HO Jr, Sandberg AA: Cytogenetic studies in primary gastric cancer. Cancer Genet Cytogenet 1986;22:295–307.
3. Ferti-Passantonopoulou AD, Panani AD, Vlachos JD, et al.: Common cytogenetic findings in gastric cancer. Cancer Genet Cytogenet 1987;24:63–73.
4. Ito H, Oda N, Ito M, et al.: Cytogenetic analysis of the human gastric carcinoma cell line TMK-1. Hiroshima J Med Sci 1989; 38:121–124.
5. Tahara E: Genetic alterations in human gastrointestinal cancers. Cancer 1995;75:1410–1417.
6. Hashimoto N, Ichikawa D, Arakawa Y, et al.: Frequent deletions of material from chromosome arm 1p in oligodendroglial tumors revealed by double-target fluorescence in situ hybridization and microsatellite analysis. Genes Chromosomes Cancer 1995;14: 295–300.
7. Matsumura K, Kallioniemi A, Kallioniemi O, et al.: Deletion of chromosome 17p loci in breast cancer cells detected by fluorescence in situ hybridization. Cancer Res 1992;52:3474–3477.
8. Kallioniemi OP, Kallioniemi A, Kurisu W, et al.: *ERBB2* amplification in breast cancer analyzed by fluorescence in situ hybridization. Proc Natl Acad Sci USA 1992;89:5321–5325.
9. Sauter G, Carroll P, Moch H, et al.: c-*myc* copy number gains in bladder cancer detected by fluorescence in situ hybridization. Am J Pathol 1995;146:1131–1139.
10. Evan GI, Wyllie AH, Gilbert CS, et al.: Induction of apoptosis in fibroblasts by c-*myc* protein. Cell 1992;69:119–128.
11. Nomura N, Yamamoto T, Toyoshima K, et al.: DNA amplification of the c-*myc* and c-*erb* B-1 genes in a human stomach cancer. Jpn J Cancer Res 1986;77:1188–1192.
12. Ranzani GN, Pellegata NS, Previderè C, et al.: Heterogenous protooncogene amplification correlates with tumor progression and presence of metastases in gastric cancer patients. Cancer Res 1990;50:7811–7814.
13. Mor O, Ranzani GN, Ravia Y, et al.: DNA amplification in human gastric carcinomas. Cancer Genet Cytogenet 1993;65:111–114.
14. Nakata B, Onoda N, Chung YS, et al.: Correlation between malignancy of gastric cancer and c-*myc* DNA amplification or overexpression of c-*myc* protein (in Japanese). Jpn J Cancer Chemother 1995;22:Supplement II:176–179.
15. Onoda N, Maeda K, Chung YS, et al.: Overexpression of c-*myc* messenger RNA in primary and metastatic lesions and carcinoma of the stomach. J Am Coll Surg 1996;182:55–59.

16. Japanese Research Society for Gastric Cancer (eds): ''Japanese Classification of Gastric Carcinoma.'' Tokyo: Kanehara & Co Ltd, 1995.
17. Pinkel D, Strume T, Gray JW: Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci USA 1986;83:2934–2938.
18. Rao PH, Mathew S, Lauwers G, et al.: Interphase cytogenetics of gastric and esophageal adenocarcinomas. Diagn Mol Pathol 1993; 2:264–268.
19. Abe K: Numerical chromosomal aberrations detected by fluorescence in situ hybridization (FISH) in gastric cancer (in Japanese). J Jpn Soc Cancer Ther 1995;30:897–904.
20. Waldman FM, Carroll PR, Kerschmann R, et al.: Centromeric copy number of chromosome 7 is strongly correlated with tumor grade and labeling index in human bladder cancer. Cancer Res 1991;51:3807–3813.
21. Suzuki S, Tenjin T, Shibuya T, et al.: Chromosome 17 copy numbers and incidence of p53 gene deletion in gastric cancer cells. Dual color fluorescence in situ hybridization analysis. J Nippon Med Sch 1997;64:22–29.